Effect of the methoxyiminoacetamide fungicide, SSF129, on respiratory activity in *Botrytis cinerea*

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Abstract: (*E*)-2-Methoxyimino-*N*-methyl-2-[2-(2,5-dimethylphenoxymethyl)phenyl]acetamide (SSF129) has been developed as a broad-spectrum systemic fungicide for control of cereal and fruit diseases. This compound inhibited NADH-oxidation by submitochondrial particles from mycelial cells of *Botrytis cinerea*, with an EC_{50} value of 14.5 nM, due to blockage of electron transport through the cytochrome bc_1 complex in the mitochondrial respiratory chain. However, SSF129 did not suppress, but rather increased, oxygen consumption by mycelial cells of the fungus. This was because mycelial cells contain an alternative oxidase protein and the cells have the ability to rapidly switch electron flux from the main cytochrome pathway to the alternative pathway on blockage of the former by SSF129. The alternative pathway of the mycelia seems not to be operative when the cytochrome pathway is functional. Naturally occurring flavonoids inhibited the alternative oxidase of the mycelial cells in a dose-dependent manner, with EC_{50} values of 68.4 μM for flavone and 63.7 μM for flavanone. These observations suggested that plant components play an important role in control of gray mould by SSF129.

Keywords: SSF129; *Botrytis cinerea*; β-methoxyacrylates; respiration; alternative oxidase; cytochrome bc_1 complex

1 INTRODUCTION

Increasing incidence of resistance to commercial systemic fungicides has prompted synthetic chemists to search for natural products potentially useful as fungicidal lead compounds. The structural modification of naturally occurring (*E*)-β-methoxyacrylates which are produced by various fungi and bacteria has been extensively investigated in the search for candidates for use as fungicides.[1,2] Among the compounds belonging to this category, azoxystrobin and kresoxim-methyl have recently been produced commercially as broad-spectrum fungicides for crop protection. Similarly to the natural strobilurins, these fungicides act as specific inhibitors of the cytochrome bc_1 complex in the mitochondrial respiratory chain.[3,4]

(*E*)-2-Methoxyimino-*N*-methyl-2-[2-(2,5-dimethylphenoxymethyl)phenyl]acetamide (SSF129) has been successfully developed as a broad-spectrum systemic fungicide, showing especially good control of the multiphagous gray mould *Botrytis cinerea* Pers ex Fr. From the viewpoint of chemical structure and biological performance, SSF129 belongs to the same category as (*E*)-β-methoxyacrylates.

Recently, we characterized the regulatory mechanism of respiration in *Pyricularia grisea* (Cooke) Sacc exposed to SSF126 (proposed common name metominostrobin) which has a chemical structure similar to that of SSF129.[5] Oxygen consumption by *P grisea* mycelial cells was completely suppressed following addition of SSF126, although the mycelial cells have the ability to induce the alternative pathway in response to blockage of the cytochrome pathway by this inhibitor. Interestingly, SSF129 had no effect on oxygen consumption by *B cinerea* mycelial cells *in vitro*, suggesting that the respiration of *B cinerea* is regulated differently from that of *P grisea*. Here, we will describe the regulation of respiration in *B cinerea* and propose a possible mechanism by which SSF129 controls gray mould caused by this fungus.

2 MATERIALS AND METHODS

2.1 Chemicals

The following agents were purchased from Sigma (St Louis, MO): defatted bovine serum albumin (BSA), ADP, ATP and β-NADH. All other reagents were of analytical grade and were purchased from Wako Chemical Co (Kyoto, Japan). SSF129 (99% purity) was synthesized by Aburahi Laboratories, Shionogi Co, Ltd (Shiga, Japan) (Fig 1). A monoclonal

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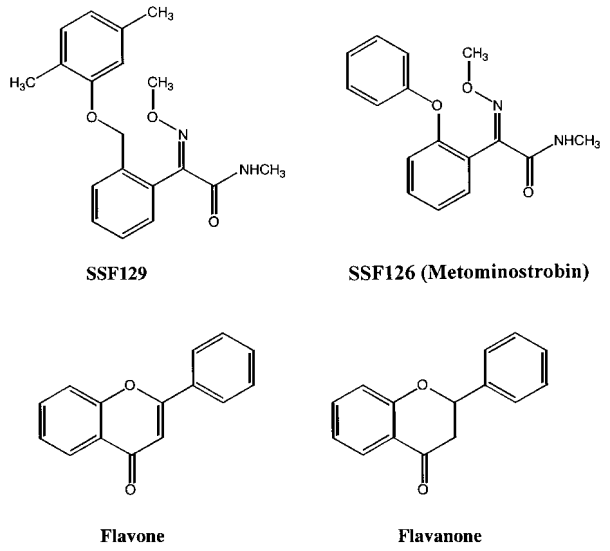

Figure 1. Structural formulae of SSF129, SSF126 (metominostrobin) and the flavone and flavanone tested.

antibody against maize alternative oxidase (AOA: PM043) was obtained from GT Monoclonal Antibodies (Lincoln, NE). Horseradish peroxidase-conjugated anti-mouse IgG and the ECL system were purchased from Amersham (Tokyo). Potassium cyanide was dissolved in distilled water, and other effectors were dissolved in dimethyl sulfoxide.

2.2 Fungus and culture conditions

B cinerea was grown on PSA (potato sucrose agar) medium under a black-light blue lamp (FL20S BLB, wavelength 352–440 nm, 20 W, Toshiba Co, Tokyo, Japan) at 25 °C for six days. Conidia obtained were transferred to 5-litre flasks containing 2 litres of potato dextrose liquid medium (PDL medium, Nissui Co, Tokyo, Japan) at a concentration of 1×10^5 conidia ml^{-1} and grown aerobically in an orbital shaker (OSI-502, Tokyo Rika Co, Tokyo, Japan) for 24 h at 25 °C and 125 rev min^{-1}.

2.3 Preparation of mitochondria and submitochondrial particles

Mycelial cells (20 g fresh weight) from a one-day-old culture of *B cinerea* were harvested on filter paper (No 2, Toyo Co, Tokyo, Japan) under vacuum and washed three times with HEPES-Tris buffer (20 mM; pH 7.2). The harvested cells were homogenized with sea sand (35 μm) in isolation medium composed of sucrose (250 mM), potassium chloride (10 mM), EDTA (5 mM), HEPES-Tris buffer (20 mM; pH 7.2) and BSA (1.5 g $litre^{-1}$). The homogenate was centrifuged at 1,500 g for 10 min at 4 °C, and the supernatant was retained. The precipitate obtained was resuspended in isolation medium and recentrifuged under the above conditions. The supernatant was combined and centrifuged at 10 000 g for 20 min. The resulting precipitate was washed with the isolation medium without BSA. The mitochondrial fraction was finally suspended in a small volume of sucrose (250 mM) and HEPES-Tris buffer (20 mM; pH7.2) to a concentration of 20–30 mg protein ml^{-1}, and used as the mitochondrial sample. The submitochondrial particles of *B cinerea* were prepared by the method of Mizutani *et al*.[5] Protein was determined by the method of Bradford,[6] using BSA as the standard.

2.4 Measurement of oxygen consumption

The rate of oxygen consumption was measured polarographically at 25 °C in a sealed vessel with a circulating water bath, using a Clark-type oxygen electrode (YSI-5300, Yellow Spring Instrument Co, OH). *B cinerea* mycelial cells (15 mg fresh weight) from one-day-old cultures, or the submitochondrial particles (0.6 mg protein), were suspended in HEPES-Tris buffer (20 mM; pH 7.2; 3 ml) saturated with air. For measurement of submitochondrial oxygen consumption, NADH (1 mM) was used as a respiratory substrate. Effectors were added to the suspensions of mycelial cells or submitochondrial particles at appropriate concentrations. Samples treated only with solvent served as controls. The rate of oxygen consumption was calculated from derivative recordings of oxygen electrode signals 2 min after addition of effectors. EC_{50} values were determined from log probability plots. For measurement of ADP/O value, the *B cinerea* mitochondria were suspended in air-saturated medium composed of sucrose (250 mM), potassium chloride (10 mM), KH_2PO_4 (8 mM), magnesium chloride (5 mM) and HEPES-Tris buffer (20 mM; pH 7.2). The values were determined from the derivative recordings 1 min after addition of ADP (30 μM). The oxygen electrode was calibrated with air-saturated distilled water assuming 240 μM O_2 at 25 °C.

2.5 Spectral measurement

Inhibition of mitochondrial electron transport was evaluated spectroscopically by measuring the redox state of cytochromes with a Hitachi 557 spectrophotometer (Hitachi Co, Tokyo, Japan) in the split-beam scanning mode with the wavelength scale at 530–630 nm. Submitochondrial particles suspended in air-saturated HEPES-Tris buffer (20 mM; pH 7.2; 3 ml) to a concentration of 2.0 mg protein ml^{-1} were placed in sample and reference cuvettes. After the baseline had been recorded, succinate (5 mM) was added to the sample cuvette to reduce the cytochromes in the presence or absence of SSF129 [10 μg (mg protein)$^{-1}$]. When the reduction was at its maximum, the difference spectrum of succinate-reduced minus air-oxidized cytochromes was recorded at a bandwidth of 1 nm.

2.6 Immunoblotting

Mitochondrial proteins from *B cinerea* mycelial cells were treated according to the method of Laemmli,[7] followed by SDS-PAGE in 10–20% polyacrylamide gradient gels (Daiichi Pure Chemicals, Tokyo) at 40 mA for 1 h. The separated proteins were transferred

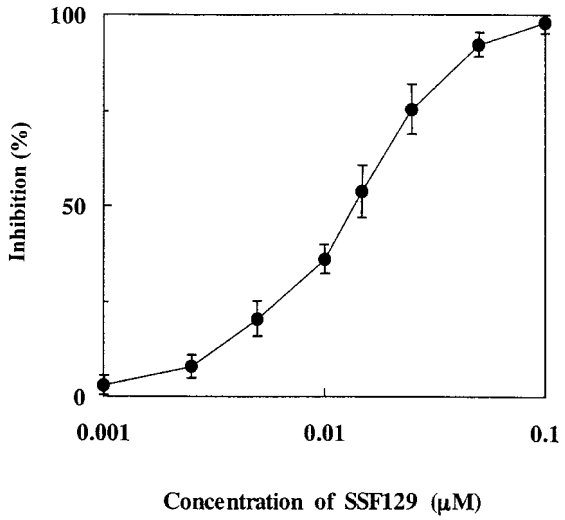

Figure 2. Inhibitory effect of SSF129 on NADH-oxidation by submitochondrial particles from *Botrytis cinerea* mycelial cells. Control activity was 219.9 nmol O_2 min^{-1} (mg protein)$^{-1}$. Results are means of three separate experiments with bars showing the standard errors.

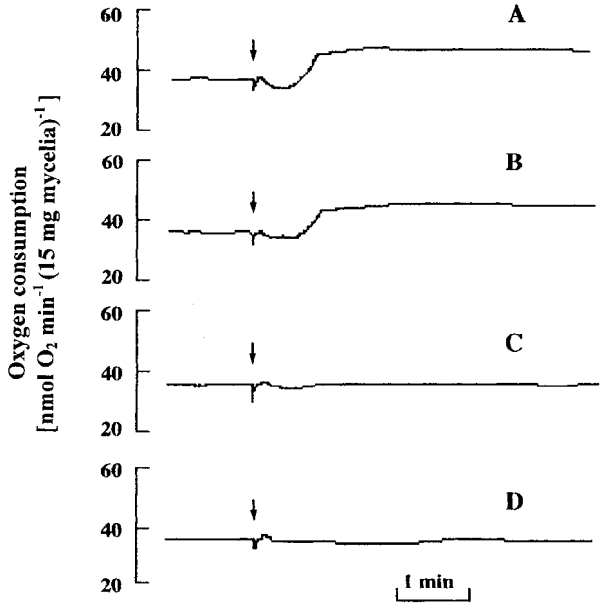

Figure 4. Effect of SSF129 on the rate of oxygen consumption by *Botrytis cinerea* mycelial cells. The arrows indicate the time at which SSF129 was added. Concentration of SSF129 was 10 μM in A, 1 μM in B and 0.1 μM in C. Control (D) activity was 37.4 nmol O_2 min^{-1} (15 mg mycelia in fresh wt)$^{-1}$.

onto PVDF membrane filters (Millipore, Tokyo) using a semi-dry electroblotter (Integrated Separation System, Natick, MA). The membranes were blocked overnight with a medium composed of Tris-HCl (20 mM; pH 7.6), sodium chloride (137 mM), Tween 20 (0.5 g litre^{-1}) and non-fat dried milk (50 g litre^{-1}) and then probed with anti-alternative oxidase antibody at a dilution of 1:30 for 1 h. The membranes were washed three times with blot rinse buffer composed of Tris-HCl (20 mM; pH 7.6), sodium chloride (137 mM) and Tween 20 (0.5 g litre^{-1}), incubated with horseradish peroxidase-linked secondary antibody at a dilution of 1:1000, and the bound antibodies were detected using the ECL chemiluminescent reagent system.

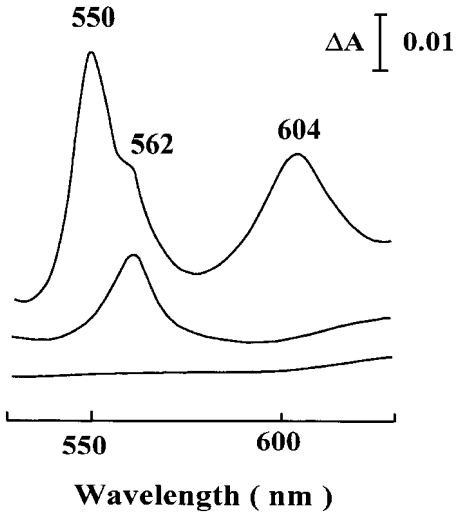

Figure 3. Difference absorbance spectra of *Botrytis cinerea* submitochondrial particles. Spectrum reduced after addition of 5 mM succinate (top trace); spectrum reduced after addition of succinate in presence of SSF129 (middle trace); baseline in bottom trace.

3 RESULTS

3.1 Effect of SSF129 on respiratory activity

SSF129 inhibited the oxygen consumption by the submitochondrial particles from *B cinerea* mycelial cells in a dose-dependent fashion when NADH was used as a respiratory substrate (Fig 2). The oxygen consumption was completely inhibited at a concentration of 0.1 μM, with an EC_{50} value of 14.5 nM. The difference spectra (reduced minus oxidized) of submitochondrial particles from mycelial cells are shown in Fig 3. Addition of succinate to submitochondrial particles led to the reduction of cytochromes c_1 (550 nm), *b* (562 nm) and *a* (604 nm) in the α-region (Fig 3, top trace). The difference spectrum of submitochondrial particles, reduced with succinate in the presence of SSF129, showed only one peak representing cytochrome *b* in the α-region, indicating that cytochromes *a* and c_1 were re-oxidized, but cytochrome *b* remained reduced (Fig 3, middle trace). These indicated that SSF129 interacted with the cytochrome bc_1 complex of the respiratory chain as described for other derivatives of β-methoxyacrylate.[4,5,8] Thus, SSF129 functions as a respiratory inhibitor. However, SSF129 could not suppress the oxygen consumption by *B cinerea* mycelial cells, and addition of the compound rather resulted in enhancement of the rate of consumption (Fig 4).

3.2 Respiratory characteristics of mycelial cells

To characterize the elevated oxygen consumption following the addition of SSF129, ADP/O values were evaluated with the mitochondria from mycelial cells incubated with SSF129 (Table 1). Control mitochon-

Table 1. Effects of respiratory inhibitors on ADP/O values of mitochondria from *Botrytis cinerea* mycelial cells treated with SSF129

	ADP/O value (±SEM)	
Inhibitor	*Succinate*	*Malate*
None	0	0.74(±0.02)
Potassium cyanide	0	0.75(±0.04)
SHAM	0	0

Results are means and standard errors from three separate experiments. *Botrytis cinerea* mycelial cells were incubated with 10 μM SSF129.

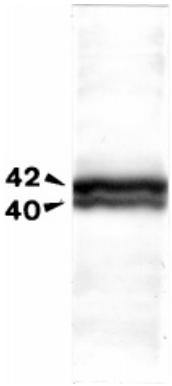

Figure 6. Western blotting analysis of alternative oxidase protein in mitochondria from *Botrytis cinerea* mycelial cells grown under normal conditions. Arrows indicate estimated molecular masses of the bands in kD. 10 μg of mitochondrial protein was applied to the lane.

dria gave ADP/O values of 2.16 for 5 mM malate and 1.33 for 15 mM succinate (data not shown), indicating that three phosphorylating sites are operative in the mitochondria. With mitochondria from the mycelial cells incubated with SSF129, the ADP/O value was very low (0.74 instead of 2.16 in controls), and remained unchanged following addition of potassium cyanide. Furthermore, the mitochondria gave an ADP/O value of zero under conditions of both malate oxidation in the presence of salicylhydroxamic acid (SHAM) and succinate oxidation. These indicated that the oxygen consumption following addition of SSF129 is due to electron flow through the alternative cyanide-resistant respiratory pathway.

For assessment of the activity and capacity of the alternative pathway in *B cinerea* mycelial cells, titration with SHAM was performed in the absence or presence of potassium cyanide according to the method of Møller *et al*.[9] In the absence of potassium cyanide, the rate of oxygen consumption was 37.4 nmol O_2 min^{-1} (15 mg mycelia fresh weight)$^{-1}$ (Fig 5) and was not affected by any concentration of SHAM tested. This suggested that there was no electron flow through the alternative pathway when the cytochrome pathway was functional. In contrast, oxygen consumption markedly increased in the presence of potassium cyanide, as observed with SSF129, reaching 47.8 nmol $O_2 min^{-1}$ (15 mg mycelia fresh weight)$^{-1}$. The capacity of the alternative pathway in the mycelial cells was 44.3 nmol $O_2 min^{-1}$ (15 mg mycelia fresh wt)$^{-1}$ from the calculation of the rate of oxygen consumption in the presence of potassium cyanide minus the residual consumption.

Mitochondrial protein was immunoblotted with the alternative oxidase monoclonal antibody to determine whether the oxidase is inductive or constitutive in *B cinerea* mycelial cells (Fig 6). In the mitochondria from mycelial cells grown under normal conditions, two polypeptide bands with apparent molecular masses of 40 kD and 42 kD were reactive with the antibody, indicating that the alternative oxidase exists as a

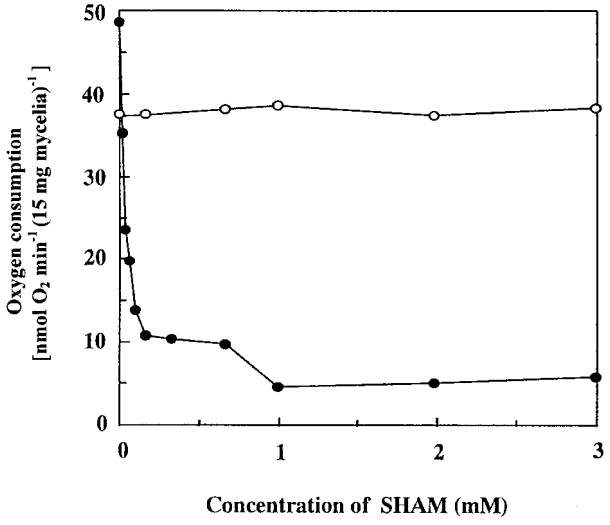

Figure 5. Effect of salicylhydroxamic acid (SHAM) on the rate of oxygen consumption by *Botrytis cinerea* mycelial cells (●) in the presence or (○) absence of 0.5 mM potassium cyanide. Results are means of three separate experiments. Standard errors were within 10%.

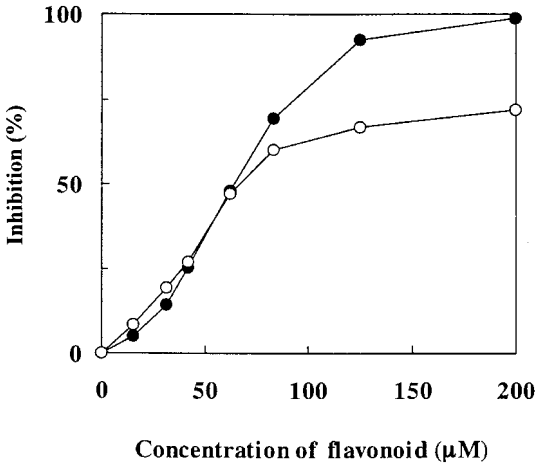

Figure 7. Inhibitory effect of flavonoids on the alternative pathway in *Botrytis cinerea* mycelial cells. Open circles indicate flavone, closed circles indicate flavanone. Results are means of four separate experiments. Standard errors were within 10%.

constitutive enzyme in *B cinerea* mycelial cells. This explained why SSF129 could not suppress respiration of *B cinerea* even immediately after addition (Fig 2). The finding that the monoclonal antibody reacts with two closely spaced polypeptide bands in *B cinerea* mitochondria is similar to the situation in *Neurospora crassa* Shear et B Dodge, in which the alternative oxidase is encoded by a single gene.[10] From this, there appear to be a single polypeptide which is modified to give the multiple bands in the immunoblot, although we have not identified the gene encoding the alternative oxidase protein of *B cinerea*.

3.3 Effect of flavonoids on alternative oxidase

Although SSF129 is a potent inhibitor of the cytochrome pathway in the mitochondrial respiratory chain, it is not possible to explain its fungicidal activity only by the inhibitory potency because of the existence of the alternative pathway, bypassing the cytochrome pathway, in *B cinerea* mycelial cells.

Phenylpropanoid substances including flavonoids, furanocoumarins and lignin are known to accumulate in response to infection with pathogenic fungi.[11–13] Furthermore, flavonoids possess several biological properties and their high chemical reactivity is expressed in their ability to catalyse electron transport[14,15] as well as to scavenge free radicals.[16,17] To clarify the fungicidal mechanism of SSF129 against *B cinerea*, the inhibitory potencies of flavonoids against the activity of the alternative oxidase in *B cinerea* were evaluated. Both flavone and flavanone (Fig 1) inhibited the activity of the alternative oxidase in *B cinerea* mycelial cells in a dose-dependent-fashion, with EC_{50} values of 68.4 μM and 63.7 μM, respectively (Fig 7). Comparable results were obtained for them in experiments using the mitochondria from *B cinerea* mycelial cells, giving EC_{50} values of 53.7 μM for flavone and 44.1 μM for flavanone (data not shown). In contrast, other flavonoids tested such as quercetin and naringenin had no inhibitory effect on the activity of the alternative pathway at a concentration of 250 μM.

4 DISCUSSION

SSF129 strongly inhibited electron flux through the cytochrome pathway in the mitochondrial respiratory chain of *B cinerea*. Derivatives of β-methoxyacrylate such as SSF126 (metominostrobin), azoxystrobin and myxothiazol are known to bind to the Qo site of the cytochrome bc_1 complex and to block electron flux in the mitochondrial respiratory chain. SSF129 also inhibited the mitochondrial respiration in the manner expected from its structural similarity to other derivatives of β-methoxyacrylate. However, SSF129 did not suppress, but rather increased, the oxygen consumption by *B cinerea* mycelial cells. Furthermore, the oxygen consumption following addition of SSF129 was resistant to potassium cyanide and gave ADP/O values of 0.74 for malate and zero for succinate oxidation. These observations indicated that oxygen consumption was due to the alternative cyanide-resistant respiration which branches from the main cytochrome pathway at the level of the ubiquinone pool and terminates with an alternative oxidase as previously described by Hayashi *et al.*[18]

In fungi such as *N crassa* and *P grisea*, the alternative oxidase protein is present at very low levels or is undetectable under normal growth conditions, but these fungi are able to induce the protein in response to blockage of the cytochrome pathway or inhibition of mitochondrial protein synthesis.[10,19–21] In contrast, *B cinerea* mitochondria contain detectable levels of the alternative oxidase protein under normal growth conditions. Thus, the respiration of *B cinerea* is regulated in a different way from that of other fungi such as *N crassa* and *P grisea*, and the regulatory system of respiration enables *B cinerea* to switch promptly to the alternative pathway following blockage of the cytochrome pathway by SSF129. The alternative pathway is not totally non-phosphorylating because the potential energy is retained at complex I when NAD-linked substrates are used as respiratory substrates.[22,23] Consequently, switching of respiration to the alternative pathway allows *B cinerea* to survive exposure to SSF129.

Recently, we proposed a mechanism of control of rice blast by SSF126.[20] The mitochondria from *P grisea* (a pathogen of rice blast) did not contain the alternative oxidase protein under normal growth conditions, unlike *B cinerea*. However, the blockage of the cytochrome pathway by SSF126 led to the induction of the oxidase in *P grisea* mycelial cells and the induction was mediated by active oxygen (O_2^-) produced in response to blockage of the cytochrome pathway. On the other hand, flavonoids blocked the induction of the alternative oxidase by scavenging the oxygen radical. From these observations, the following fungicidal mechanism of SSF126 against *P grisea* was deduced; flavonoids in plants inhibit the SSF126 dependent induction of the alternative oxidase by scavenging the oxygen radical and lead to complete suppression of fungal respiration. However, the fungicidal mechanism proposed for *P grisea* was not applicable for *B cinerea* because the alternative oxidase protein was constitutively present in *B cinerea* mitochondria.

Some flavonoids, (flavone and flavanone) which are major phenolic constituents of naturally occurring substances as antioxidants, inhibited the activity of the alternative oxidase of *B cinerea* in a dose-dependent fashion, suggesting that plant components also play an important role in the control of gray mould. In contrast, other flavonoids such as quercetin and naringenin showed no inhibition of the function of the alternative oxidase, indicating that the inhibitory potency of flavonoids against the alternative oxidase of *B cinerea* is dependent on their chemical structure. In field trials, SSF129 showed a good control efficacy against *B cinerea* on tomatoes, eggplants, cucumbers,

lettuces and kidney beans. These plants may contain common flavonoid components inhibitory to the alternative oxidase of *B cinerea*.

In conclusion, the alternative pathway is regulated in at least two different ways in fungi. Some fungi show induction of the alternative oxidase protein in response to impairment of the main cytochrome pathway. On the other hand, others express this protein constitutively in addition to the cytochrome pathway under normal growth conditions. Flavonoids in plants could exhibit two types of interaction with the alternative pathway in the fungicidal mechanism of derivatives of β-methoxyacrylates which block electron flux through the cytochrome bc_1 complex in the mitochondria. The first could be characterized as inhibition of the induction of the alternative oxidase protein as described in *P grisea*, and the second as inhibition of the activity of the alternative oxidase as in *B cinerea*. The regulatory mechanism of the alternative pathway in pathogenic fungi probably decides which functions of flavonoids are useful for interference of the pathway. The functions of flavonoids would contribute to in-vivo activity and overall performance of fungicides belonging to the category of β-methoxyacrylate.

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REFERENCES

1 Beautement K, Clough JM and Fraine PJ, Fungicidal β-methoxyacrylates: from natural products to novel synthetic agricultural fungicides. *Pestic Sci* **31**:499–519 (1991).

2 Clough JM, The strobilurins, oudemansins, and myxothiazol, fungicidal derivatives of β-methoxyacrylic acid. *Natural Product Rep* 565–574 (1993).

3 Becker WF, Von Jagow G, Anke T and Steglich W, Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc_1 segment of the respiratory chain with an *E*-β-methoxyacrylate system as common structural element. *FEBS Lett* **132**:329–333 (1981).

4 Mansfield RW and Wiggins TE, Photoaffinity labelling of the β-methoxyacrylate binding site in bovine heart mitochondria cytochrome bc_1 complex. *Biochim Biophys Acta* **1015**:109–115 (1990).

5 Mizutani A, Yukioka H, Tamura H, Miki N, Masuko M and Takeda R, Respiratory characteristics in *Pyricularia oryzae* exposed to a novel alkoxyiminoacetamide fungicide. *Phytopathology* **85**:306–311 (1995).

6 Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**:248–254 (1976).

7 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **227**:680–685 (1970).

8 Mizutani A, Miki N, Takenaka H and Tamura H, Binding characteristics of methoxyiminoacetamide derivatives to cytochrome c reductase. *Pestic Biochem Physiol* **57**:119–125 (1997).

9 Møller IM, Berczi A, Van der Plas HW and Lambers H, Measurement of the activity and capacity of the alternative pathway in intact plant tissues: Identification of problems and possible solutions. *Physiol Plant* **72**:642–649 (1988).

10 Lambowitz AM, Sabourin JR, Bertrand H, Nickels R and McIntosh L, Immunological identification of the alternative oxidase of *Neurospora crassa* mitochondria. *Mol Cell Biol* **9**:1362–1364 (1989).

11 Kuhn DN, Chappel J, Boudet A and Hahlbrock K, Induction of phenylalanine ammonia-lyase and 4-coumarate: Co A ligase mRNA in cultured plant cells by UV light or fungal elicitor. *Proc Natl Acad Sci* **81**:1102–1106 (1984).

12 Hahlbrock K, Knobloch KH, Kreuzaler F, Potts JRM and Wellmann E, Coordinated induction and subsequent activity changes of two groups of metabolically interrelated enzymes. Light-induced synthesis of flavonoid glycosides in cell suspension cultures of *Petroselinum hortense*. *Eur J Biochem* **61**:199–206 (1976).

13 Dyer WE, Henstrand JM, Handa AK and Herrmann KM, Wounding induces the first enzyme of the shikimate pathway in solanaceae. *Proc Natl Acad Sci* **86**:7370–7373 (1989).

14 Dons RF and Doughty CC, Isolation and characterization of aldose reductase from calf brain. *Biochim Biophys Acta* **452**:1–12 (1976).

15 Mukohara Y, Nakabayashi S and Higashida M, Quercetin, an energy transfer inhibitor in photophosphorylation. *FEBS Lett* **85**:215–218 (1978).

16 Baumann J, Wurm G and Bruchhausen F, Hemmung der prostaglandinsynthetase durch flavonoide und phenoldervate im vergleich mit deren O_2^- radikalfangereigenschaften. *Arch Pharm (Weinheim)* **313**:330–337 (1980).

17 Naim M, Gestetner B, Bondi A and Birk Y, Antioxidative and antihemolytic activities of soybean isoflavones. *J Agric Food Chem* **24**:1174–1177 (1976).

18 Hayashi K, Watanabe M, Tanaka T and Uesugi Y, Cyanide-insensitive respiration of phytopathogenic fungi demonstrated by antifungal joint action of respiration inhibitors. *Nihon Noyaku Gakkaishi (J Pestic Sci)* **21**:399–403 (1996).

19 Edwards DL, Rosenberg E and Maroney PA, Induction of cyanide-insensitive respiration in *Neurospora crassa*. *J Biol Chem* **249**:3551–3556 (1974).

20 Mizutani A, Miki N, Yukioka H, Tamura H and Masuko M, A possible mechanism of control of rice blast disease by a novel alkoxyiminoacetamide fungicide, SSF126. *Phytopathology* **86**:295–300 (1996).

21 Yukioka H, Tanaka R, Inagaki S, Katoh K, Miki N, Mizutani A, Masuko M and Kunoh H, Mutants of the phytopathogenic fungus *Magnaporthe grisea* deficient in alternative, cyanide-resistant, respiration. *Fungal Genet Biol* **22**:221–228 (1997).

22 Moore AL, Bonner WD and Rich PR, The determination of the proton motive force during cyanide-insensitive respiration in plant mitochondria. *Arch Biochem Biophys* **186**:298–306 (1978).

23 Passam HC and Palmer JM, The ATPase activity of Jerusalem-artichoke mitochondria and submitochondrial particles. *Biochim Biophys Acta* **305**:80–87 (1973).